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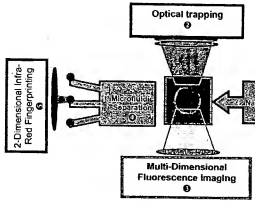
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR THE ANALYSIS OF CELLS



(57) Abstract: The present invention provides an improved method of analysing and obtaining reliable data about the plasma membrane of single cells, using a microfluidic cell analyser. The microfluidic cell analyser of the invention comprises a single cell trap, a manipulator arranged to manipulate the outer surface of a cell in the trap, a detection zone in communication with the single cell trap and a detector.

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Method for the analysis of cells

The present invention relates to a microfluidic cell analyser.

The analysis of populations of cells has provided important information and has led to a number of important medical, clinical and scientific developments. However, cells within a given population are heterogeneous. This leads to problems with the use of population averaged cell preparation techniques and has prompted the need for alternative ways of analysing cells. In particular, this has lead to the development of single cell analytic methods.

Single cell analytic methods are believed to overcome disadvantages associated with population averaged analytic methods. In particular, such methods allow more accurate information to be obtained, allowing a better understanding of cell responses to therapeutic interventions and disease states. Single cell analysis therefore has the potential to become an important tool for aiding and enabling the development of predictive and preventative medicine tailored to individual patients.

Microfluidic systems have previously been used to perform single cell analysis. However, such analysis has concentrated on the intracellular components of the cell. The analytic methods of the art have therefore required the location of a single cell into a separation channel, lysis of the cell, separation of the cellular components and analysis of the intracellular component of interest by capillary electrophoresis.

While the analysis of intracellular components of a cell may provide interesting biological information, the interaction of a cell with its surrounding environment is primarily determined by the plasma membrane.

The plasma membrane defines the extent of a cell and moderates the interaction of the cell with its external environment. It performs many roles including acting as a filter, allowing active transport, controlling the entry and exit of substances into and out of the cell, generating differences in ion concentrations between the interior and exterior of the cell and sensing external signals.

The plasma membrane is the subject of many disease states and a target for many therapeutic interventions. The plasma membrane is involved in the transduction of a large number of signaling pathways, is the entry and exit route for all cellular components and is the means by which cells interact with and communicate with their environment. The plasma membrane is therefore an important target for single cell analysis.

The plasma membrane is a lipid bilayer, said bilayer being composed of phospholipids, cholesterol and glycolipids. In addition, the plasma membrane comprises a numerous additional components such as proteins, glycoproteins etc. The plasma membrane therefore contains many thousands of proteins as well as a complex mix of lipids. Current analysis methods of the plasma membrane are broadly based on one of two strategies. The conventional protein analysis method is to take an ensemble of cells from tissue or culture, homogonise them, and then separate the components. An example of this is the MALDI-TOF proteomic approach, which uses two-dimensional gels, or liquid phase separation to isolate individual proteins and mass spectrometry to identify them. The 2D gel MALDI-TOF approach is however highly unsatisfactory for membrane proteins, and has limited utility due to problems with low fidelity, insensitivity and isolation difficulties. An alternative approach for the analysis of lipid composition of plasma membranes is electrospray ionisation mass spectrometry (ESI-MS), which allows the analysis

of phospholipid structures. Similar techniques can be applied to membrane proteins however the proteins must initially be separated using a combination of isoelectric focusing (IEF) and SDS electrophoresis which often proves difficult and time consuming.

An alternative analysis method is the use of *in vivo* fluorescence microscopy. This method can be applied to single cells. However, fluorescence microscopy depends on specificity of antibody or transfection labeling. This is a major disadvantage of fluorescence microscopy and has limited the application of this technique to the examination of only two, or three proteins at one time.

There is therefore a need in the art for an improved method of analysing and obtaining reliable data about the plasma membrane of cells, in particular the plasma membrane of single cells.

The first aspect of the invention provides a microfluidic cell analyser comprising a single cell trap, a manipulator arranged to manipulate the outer surface of a cell in the trap, a detection zone in communication with the single cell trap and a detector.

The invention may be put into practice in various ways and a number of specific embodiments will be described by way of example to illustrate the invention with reference to the accompanying drawing:

Figure 1 which shows a schematic representation of the microfluidic cell analyser.

The microfluidic cell analyser as illustrated in figure 1 comprises a single cell trap, for example an optical trap (2) monitored by a multi-dimensional

fluorescence imager (3), a manipulator (not shown) such as laser dissection, a microfluidic separator (4), a detection zone (not shown) and a detector (5).

Pre-separation of the cell sample is carried out at (1) by controlled microfluidic nano-digestion. The cell is then isolated in the optical trap (2) and monitored by multi-dimensional fluorescence imaging (3). Manipulation of the cell is carried out by one or more manipulators (not shown) and the post-digestion products are separated by a microfluidic separator (4) prior to detection (5) via 2D optical finger printing or multi-dimensional fluorescence imaging.

The microfluidic cell analyser is provided for the analysis of the outer surface of a cell, particularly for the analysis of the plasma membrane of a cell.

The analyser can be used to analyse one or more proteins, glycoproteins or lipids in or associated with the plasma membrane of the cell.

In use, a single cell is introduced into the optical cell trap. For the purposes of this invention, the optical trap comprises a laser and a focusing lens. Manipulation of the cell within the optical trap is achieved by a controller which contains the focused laser beam's position, polarization, phase and profile and which is used to realize the functions given above. This controller can be included on the low magnification side of the lens where the laser beam is collimated.

The position of the cell within the optical trap is monitored by an imager, such as a multi-dimensional fluorescence imager, which provides information regarding the status of the cell, this information being fed back to the controller.

The term "optical trapping" refers to the process whereby cells of a high dielectric constant are naturally attracted to regions of high electric field, for example the maximum electric field produced in the focus of a laser beam. Alternatively optical trapping is effected by forming a dark spot in the centre of the focused beam to trap cells (with appropriate dielectric properties) which are repelled by the field, wherein the object is forced into the dark centre. The exact form of the beam focus e.g. to form lines or curves, can be used to trap extended or non-spherical cells.

Alternatively the cell may be trapped by other methods, such as electrostatics.

Manipulation of the cell within an optical trap can be achieved by for example using the focused laser as a controller to move the spot in three dimensions to change the position of the cell. Alternatively adjusting the separation of multiple spots can stretch or compress a cell and rotating the polarization or pattern of spots can rotate the cell. Similarly beam profiles with so-called "angular momentum" can be used to rotate the cell.

The cell is trapped to ensure that the cell remains stationary within the solvent flow, during analysis. Because of the dynamic fluid environment as well as the changing size and characteristics of the cell as it is manipulated, adaptive control is required to maintain the cell and its components in position. This is achieved for the present invention using programmable diffractive optics to dynamically control a near infra-red laser trapping beam in real time. Programmable diffractive optics is achieved with a Spatial Light Modulator (SLM), which provides a means of altering the phase, amplitude or polarization of light reflected off or passing through it under external electrical or optical control. The device is programmed with an appropriate computer generated hologram and with the aid of appropriate external optical components. The

SLM is configured to impart an arbitrary phase, polarization and amplitude distribution onto a laser beam. In this way the device allows the dynamic control of the focused profile of the laser beam in the optical trap.

Other methods of controlling the laser beam for the purpose of this invention include scanning mirrors or acousto-optic deflectors. These are capable of temporally multiplexing (scanning) the beam between multiple positions.

The programmable diffractive optics allows the use of complex beam patterns to effectively trap the whole of the cell and its contents as the manipulation process proceeds. The set-up is inherently adaptive to be able to compensate for changes in the optical properties of the flowing cell medium and the programmable nature permits the cell to be moved or rotated. This feature interacts with active control of the flow dynamics to permit selective targeting of specific features on the cell membrane. Interactive use of the system with an operator allows the dynamic manipulation of the cell, so that the selective features are positioned by rotating or translating the trapped cell appropriately into the fluid flow.

The trapping and manipulation of the single cell may be combined with an imager such as online multi-dimensional fluorescence imaging (MDFI: resolving fluorescence in 2 or 3 spatial dimensions and with respect to some or all of lifetime, wavelength and polarisatrion) to provide an interactive system for manipulating and monitoring the cell. This provides interactive feedback to enhance the selective targeting of specific features in the cell membrane. The online multi-dimensional fluorescence imaging can use either endogenous autofluorescence or appropriate fluorescence labels to provide an interactive system for manipulating and monitoring the cell. Alternatively or in addition, the online multi-dimensional fluorescence imaging can provide an interactive

system that may also be coupled to the selective dissolution of the plasma membrane. Furthermore, the online multi-parameter fluorescence imaging can be used to monitor the process and assist with the readout of the analytes sperted by the microfluidics system.

Cells can be introduced into the microfluidic cell analyser through an input reservoir. They can be moved through the analyser using suitable forces, such as hydrodynamic forces and/or electrokinetic forces, through a microchannel network in the analyser to a suitable position where they can be optically trapped. The microchannels have dimensions (channel width and channel height) so as to allow facile passage of the cell through the channel without interaction with microchannel surfaces and walls.

The single cell can be introduced into the trap using an aspiration assembly in conjunction with micromanipulators. This will allow the cell to be transferred from its growth medium to the microfluidics device where lamellar streams will then be used to deliver the cell to the optical trap.

The microfluidic cell analyser can additionally comprise a microfluidic separator located between the single cell trap and the detection zone. Separation of the components subsequent to manipulation can be carried out by size, isoelectric focussing, mass and/or mass/charge. Alternatively the cell can be analysed in the optical trap.

The outer surface of the trapped cell undergoes manipulation by a manipulator. In particular, the plasma membrane is manipulated by the manipulator. Manipulation can be carried out by physical or chemical means. In particular, the manipulator can cause the outer surface of the cell to be exposed to an enzyme (such as a lipase), a lipid, a detergent, sonication and/or physical

agitation (e.g. laser dissection). The chemical agents can be delivered to defined locations (subcellular microdomains) on the membrane surface using multiple laminar streams within the microchannel. By controlling both the position and angular orientation of the cell relative to the reagent stream it is possible to either digest the plasma membrane in its entirety or selectively target subcellular microdomains thereby providing spatial information regarding the distribution of chemical moieties within the plasma membrane. Material released from the membrane is collected in continuous flow and directed downstream for separation and analysis. Alternatively, the biology of the cell may be manipulated to promote areas of stress within the plasma membrane (i.e. via the initiation of exocytosis) by the delivery of appropriate chemical agents. The cell may further be incubated with one or more hormones, proteins, etc.

In particular a small plug (10 pL –10 nL) of material that can digest the membrane can be introduced into the analyser, for example using electrokinetic control and/or 'tee-injectors' upstream of the optical trap. When the plug contacts the cell, the cell is rotated to allow the digested material to be released and motivated downstream towards the microfluidic separator.

The plasma membrane may be manipulated over the whole of the external cell surface. Alternatively, a portion of the plasma membrane may be manipulated. In particular, manipulation may be directed to the lipids and/or proteins comprising the plasma membrane.

The analytical device of the first aspect of the invention is directed to the analysis of the outer cell surface, more specifically to the plasma membrane. The manipulation of the plasma membrane does not result in the lysis of the cell. It will be appreciated that disruption of localized areas of the plasma

membrane may result in the formation of a weakened area or a disruption of the membrane through which some of the cell contents may pass. However, such increase in the permeability of the membrane does not directly lead to full lysis of the cells.

Once manipulation of the cell has been completed, the cell can subsequently be lysed and the internal contents of the cells separated from the plasma membrane contents. The separated plasma membrane can then be further digested as required. Alternatively, the cell can be retained in an intact form and the cell separated from any digestion products.

The analyser comprises a detector for detecting the outer surface of the single cell, or components of the outer surface of the single cell. The cell is preferably detected by multiparameter fluorescence imaging and/or optical finger printing.

In particular, the plasma membrane and/or components thereof can be analysed using multiparameter fluorescence imaging (MDFI). MDFI is realized using a high-speed quasi-wide-field multiphoton microscope to provide rapid optical sectioning together with the ability to rapidly acquire excitation and emission spectral profiles as well as fluorescence lifetime data. The MDFI offers at least three important capabilities. First, using appropriate fluorescence labels (either genetically expressed or tagged with antibodies), it enables the direct observation of specific components in the cell membrane and their tracking through the microfluidic separation system. Secondly, the use of multi-photon excitation combined with spectrally resolved FLIM provides unprecedented contrast of autofluorescence, permitting the entire process to be controlled and monitored without using exogenous fluorescence labels that could compromise

some samples. Finally, the MDFI provides one way to achieve the optical read-out of the microfluidic separation, distinguishing different proteins etc.

Alternatively, or in addition optical readout can be obtained with single molecule sensitivity using multi-dimensional fluorescence imaging and/or optical fingerprinting technologies. While MDFI essentially probes the spectroscopy associated with the electronic energy level structure, the optical fingerprinting technology is a vibrational spectroscopic tool that can directly resolve the individual bonding patterns in molecules.

Optical fingerprinting is achieved using an optical analog of 2D NMR. This optical analog uses two infra-red laser beams to excite two vibrations, and the vibrational coupling can then be monitored in a variety of ways. In a preferred feature of the invention, detection is obtained using DOubly Vibrationally Enhanced (DOVE) spectroscopy. DOVE reads out the vibrational coupling using a third laser pulse. The invention also encompasses extensions of DOVE such as TRIVE which use three infra-red pulses to excite three vibrations, and a fourth pulse to read out the coupling. This measurement of coupling is analogous to NOESY or COESY, NMR methods that measure spin-spin coupling. The vibrational coupling spectrum is projected over a multi-dimensional spectral space, one for each IR laser beam, and also one time dimension for the timing between each IR pulse. In this way the overdense and congested IR spectrum is thinned by looking at couplings only and being projected over a higher dimensional space, as is the case in 2D NMR.

The microfluidic analyser may comprise two or more single cell traps and/or two or more detection zones. This allows a high through put approach to single cell analysis. In particular, one detector (for example a multiparameter fluorescence arrangement) can be used to track two or more single cell traps

and/or two or more detection zones. The outputs of the multiple detection zones can be combined to allow high throughput read out via optical fingerprinting. This approach allows the analysis of large quantities of one or more plasma membrane components very rapidly.

Single cell methods can be scaled out by providing two or more microfluidic cell analysers. The first aspect of the invention therefore encompasses an array of microfluidic cell analysers comprising two or more microfluidic cell analysers. The analysers are provided in parallel or in series.

It will be understood by the skilled person that any appropriate component can be used for the purposes of the present invention. In particular, the single cell trap, the manipulator, the detection zone or the detector can be any appropriate component.

The single cell for the purpose of the first aspect is obtained from a largely hetereogeneous or homogeneous population of cells. Such cells include mammalian or non-mammalian cells, including plant or animal cells. The cells can be isolated from a plant or animal or produced *in vitro*. The cells may be native cells, or genetically, chemically or biologically manipulated. Such cells include dendrites, mucosal cells, and epithelial cells.

The second aspect of the invention provides a method of single cell analysis comprising trapping a single cell, manipulating the outer surface of the cell and analysing the manipulated cell surface. The outer cell surface is preferably the plasma membrane.

The outer surface of the cell can be manipulated by exposure to one or more hormones, proteins, enzymes, lipids, detergents, sonication, and/or physical

agitation. The online multi-dimensional fluorescence imaging can allow the degree and position of manipulation of the outer surface of the cell to be determined. The manipulation can then be analysed by 2D optical finger printing or multi-dimensional fluorescence imaging.

In particular, the second aspect relates to a method of single cell analysis comprising pre-separation of the cell sample by controlled microfluidic nano-digestion, trapping of the cell in an optical trap, monitoring of the cell by multi-dimensional fluorescence imaging, manipulation of the cell by a manipulator, separation of the resulting components of the cell by microfluidic separation and detection via 2D optical finger printing or multi-dimensional fluorescence imaging.

All preferred features of the aspects of the invention apply to all other aspects *mutatis mutandis*.

CLAIMS

1. A microfluidic cell analyser comprising a single cell trap, a manipulator arranged to manipulate the outer surface of a cell in the trap, a detection zone in communication with the single cell trap and a detector.

2. A microfluidic cell analyser as claimed in claim 1 further comprising a microfluidic separator between the single cell trap and the detection zone.

3. A microfluidic cell analyser as claimed in claim 1 or claim 2 wherein the trap is an optical trap.

4. A microfluidic cell analyser as claimed in any one of claims 1 to 3 wherein the trap is monitored by online multi-dimensional fluorescence imaging.

5. A microfluidic cell analyser as claimed in any one of claims 1 to 4 wherein the detector provides multiparameter fluorescence imaging and/or optical finger printing.

6. A microfluidic cell analyser as claimed in any one of claims 1 to 5 wherein the detector is a high-speed quasi-wide-field multiphoton microscope.

7. A microfluidic cell analyser as claimed in any one of claims 1 to 6 wherein the detector provides analysis by DOubly Vibrationally Enhanced (DOVE) spectroscopy.

8. A microfluidic cell analyser as claimed in any one of claims 1 to 7 comprising two or more single cell traps.

9. A microfluidic cell analyser as claimed in any one of claims 1 to 8 comprising two or more detectors.

10. An array of microfluidic cell analysers comprising two or more microfluidic cell analysers as claimed in any one of claims 1 to 9.

11. A method of single cell analysis comprising trapping a single cell, manipulating the outer surface of the cell and analysing the manipulated cell surface.

12. A method as claimed in claim 11 wherein the trapped cell is monitored by online multi-dimensional fluorescence imaging

13. A method as claimed in claim 12 wherein the online multi-dimensional fluorescence imaging uses endogenous autofluorescence or fluorescence labels.

14. A method as claimed in claim 11 or claim 12 wherein monitoring of the trapped cell provides a feedback system for manipulating and monitoring the cell.

15. A method as claimed in claim 14 wherein the feedback system is coupled to the selective dissolution of the plasma membrane

16. A method as claimed in any one of claims 11 to 15 wherein the outer surface of the cell is manipulated by exposure to one or more hormones, proteins, enzymes, lipids, detergents, sonication, and/or physical agitation.

17. A method as claimed in any one of claims 11 to 16 wherein the cell is obtained from a hetereogeneous or homogeneous population of cells.

18. A method as claimed in any one of claims 11 to 17 wherein the cell is a mammalian or non-mammalian cell.

19. A method as claimed in any one of claims 11 to 18, wherein the cell is trapped in an optical trap.

20. A method as claimed in any one of claims 11 to 19 wherein the manipulated cell surface is analysed by 2D optical finger printing or multi-dimensional fluorescence imaging.

21. A method as claimed in any one of claims 11 to 20 wherein a cell sample is pre-separated by controlled microfluidic nano-digestion, prior to trapping of the cell.

22. A method as claimed in any one of claims 11 to 21 wherein the components of the cell are separated by microfluidic separation prior to analysis.

23. A method as claimed in any one of claims 11 to 22 comprising pre-separation of the cell sample by controlled microfluidic nano-digestion, trapping of the cell in an optical trap, monitoring of the cell by multi-dimensional fluorescence imaging, manipulation of the cell by a manipulator, separation of the resulting components of the cell by microfluidic separation and detection via 2D optical finger printing or multi-dimensional fluorescence imaging.

24. A microfluidic cell analyser substantially as described herein with reference to the examples and the figures.

25. A method substantially as described herein with reference to the examples and the figures.

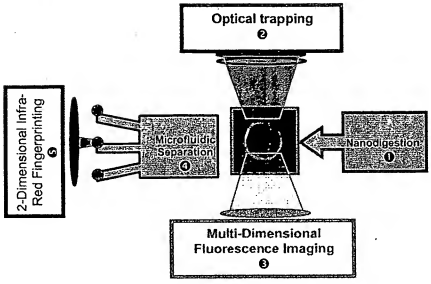
Optical trapping
❷
2-Dimensional Infra-Red Fingerprinting
❹
Microfluidic Separation
❶
Nanodigestion
❶
Multi-Dimensional Fluorescence Imaging
❸

Figure 1

INTERNATIONAL SEARCH REPORT

tional application No
/GB2005/004602

A. CLASSIFICATION OF SUBJECT MATTER
G01N15/14 G01N21/64 C12M1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, INSPEC, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99/61888 A (CALIFORNIA INSTITUTE OF TECHNOLOGY; QUAKE, STEPHEN; FU, ANNE; ARNOLD,) 2 December 1999 (1999-12-02) page 11, line 18 - page 31, line 2	1-23
X	STROMBERG A ET AL: "MANIPULATING THE GENETIC IDENTITY AND BIOCHEMICAL SURFACE PROPERTIES OF INDIVIDUAL CELLS WITH ELECTRIC-FIELD-INDUCED FUSION" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, US, vol. 97, no. 1, 4 January 2000 (2000-01-04), pages 7-11, XP002935310 ISSN: 0027-8424	1,3,11, 16-19, 21,22
Y	the whole document	4-8,10, 12,13,20

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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 March 2006	21/03/2006
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Müller, T

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZAHN M ET AL: "Fluorimetric multiparameter cell assay at the single cell level fabricated by optical tweezers" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 443, no. 3, 29 January 1999 (1999-01-29), pages 337-340, XP004259171 ISSN: 0014-5793 the whole document	4,5,8, 10,12,20
Y	KÖNIG K: "Robert Feulgen Prize Lecture. Laser tweezers and multiphoton microscopes in life sciences" HISTOCHEMISTRY AND CELL BIOLOGY, SPRINGER, BERLIN, DE, vol. 114, no. 2, August 2000 (2000-08), pages 79-92, XP002257732 ISSN: 0948-6143 the whole document	6,13
X	GOKSÖR M ET AL: "Optical manipulation in combination with multiphoton microscopy for single-cell studies" APPLIED OPTICS OPT. SOC. AMERICA USA, vol. 43, no. 25, 1 September 2004 (2004-09-01), pages 4831-4837, XP002371166 ISSN: 0003-6935 the whole document	1,3,9
Y	ZHAO W ET AL: "NONLINEAR TWO-DIMENSIONAL VIBRATIONAL SPECTROSCOPY" APPLIED SPECTROSCOPY, THE SOCIETY FOR APPLIED SPECTROSCOPY. BALTIMORE, US, vol. 54, no. 7, July 2000 (2000-07), pages 1000-1004, XP008025638 ISSN: 0003-7028 the whole document	7
X	WO 02/26114 A (BITENSKY, MARK, W; YOSHIDA, TATSURO; FRANK, MICHAEL, G; DERGANC, JURE;) 4 April 2002 (2002-04-04) page 4, paragraph 2 page 9, paragraph 4 - page 10, paragraph 3; figure 2	1
X	US 6 613 211 B1 (MCCORMICK RANDY M ET AL) 2 September 2003 (2003-09-02) column 2, lines 18-28 column 12, lines 3-23 column 13, line 32 - column 14, line 24	1,2,11, 22

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/GB2005/004602

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 24,25
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 24,25

Rule 6.2 a)

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

ational application No
/GB2005/004602

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